Cytogenetic and molecular changes in myelodysplastic syndromes

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Myelodysplastic syndromes (MDS) consist of hematologic diseases which differ in clinical features and also in cytogenetic presentation. They are characterized with abnormal development of at least one bone marrow cell line. Around 50 % patients display chromosome aberrations which have diagnostic and prognostic value. Chromosome abnormalities can be detected with different methods. We compared two of them, fluorescent in situ hybridization (FISH) and multiplex ligation-dependent probe amplification (MLPA). Despite the detection limit of MLPA, we found out that these methods can be used in routine diagnostics of MDS and the best way is their combination.

Key words: MDS, FISH, MLPA, chromosome aberrations

Cytogenetické a molekulárne zmeny pri myelodysplastickom syndróme

Myelodysplastický syndróm (MDS) je heterogénna skupina chorôb, ktorá sa líši v klinických prejavoch, ako aj v prítomnosti cytogenetických abnormalít. Pre MDS je typický abnormálny vývoj jednej alebo viacerých bunkových línií kostnej drene. Približne 50 % pacientov má prítomné chromozómové aberácie, ktoré majú prognostický a diagnostický význam. Tieto aberácie je možné vyšetriť rôznymi metódami, my sme porovnali 2 z nich, a to interfáznu fluorescenčnú *in situ* hybridizáciu (FISH) a multiplexnú od ligácie závislú amplifikáciu prób (MLPA). Aj napriek určitým limitáciám MLPA analýzy môžeme povedať, že obe tieto metódy sú vhodné na rutinnú diagnostiku a pri ich vhodnej kombinácii vieme získať najlepšie výsledky.

Kľúčové slová: MDS, FISH, MLPA, chromozómové aberácie

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Introduction

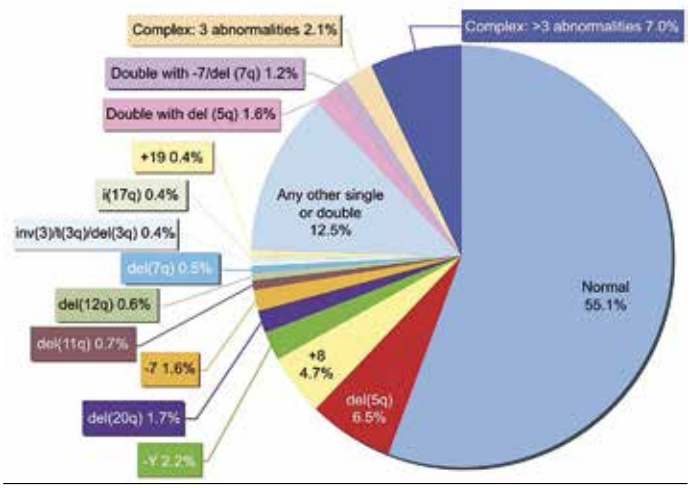
Myelodysplastic syndromes (MDS) are hematologic diseases with heterogeneous clinical manifestation. MDS is characterized by abnormal development of one or more bone marrow cell lines, resulting in one or more peripheral blood cytopenias. Patients with MDS have risk of progression to acute myeloid leukemia (AML) (1). The clinical outcome is diverse due to the complexity of genetic changes which include copy number changes (deletions, amplifications), mutations of individual genes, as well as epigenetic mutations which alter gene expression levels (2). Chromosome changes include numerical and structural aberrations, such as monosomies, trisomies, inversions or translocations, while unbalanced aberrations are more prevalent.

Table 1. MDS cytogenetic scoring system (5)

Cytogenetic prognostic subgroup	Cytogenetic abnormalities
Very good	-Y, del(11q)
Good	normal; del(5q); del(12p); del(20q); double including del(5q)
Intermediate	del(7q); +8; +19; i(17q); any other single or double abnormalities
Poor	-7; inv(3)/t(3q)/del(3q); double including -7/del(7q); complex: 3 abnormalities
Very poor	complex karyotype: > 3 abnormalities

About 50 % of patients carry clonal chromosome abnormalities. The most common are del(5q), del(7q)/–7, +8, del(11q), del(12p), del(17p), del(20q), and loss of Y chromosome (3). Some copy number changes (CNVs) have prognostic and diagnostic value (table 1). Therefore, the karyotype analysis plays crucial role in diagnostics and represents a powerful tool for establishing independent prognostic factors. Findings of cytogenetic aberrations are important part of prognostic scoring systems, e.g. International Prognostic Scoring System (4) and its revised form (5), introduced in 2012. These scoring

Figure 1. Most common cytogenetic aberrations in MDS patients (7)



systems identify abnormalities in karyotype and some clinical features which differentiate patients with MDS into prognostic subgroups.

Chromosome abnormalities can be detected by various methods, e.g. metaphase karyotyping, fluorescent *in situ* hybridization (FISH) in interphase cells or molecular analyses, such as multiplex ligation-dependent probe amplification (MLPA), array-based comparative genomic hybridization (aCGH) or whole genome sequencing (WGS). Conventional karyotyping is limited and many chromosomal aberrations cannot be detected as it is dependent on dividing cells, and it has low resolution and sensitivity. The disadvantages of FISH probes are their price and their resolution which is relatively low (in kilobases) but better than karyotyping. On the other hand, MLPA is simple, multiplex, cost-effective, PCR based, and relatively easy method and it can detect up to 50 different genomic DNA sequences (6). As the sequences detected by MLPA are only 50 – 70 nucleotides long, this method is powerful in detection of deletions or amplifications of single exones (8). In comparison to FISH, MLPA is multiplex and can detect single gene aberrations which are too small to be discovered by FISH or karyotyping. When comparing MLPA to whole genome methods, such as aCGH or WGS, MLPA is a low cost and technically simple method. Although, it cannot be used for genome-wide screening, it is a good alternative to such techniques.

Nowadays, molecular pathogenesis and the development of MDS, as well as progression to AML is still unclear (9). Many chromosomal aberrations are known, but about 50 % patients have normal karyotype. Most of the genes involved in MDS remain undiscovered. Therefore, single-gene aberration studies are currently under investigation. Research shows that around 70 % of MDS patients carry mutations, but most of them are rare (10, 11). Approximately 40 genes seem to be mutated in most patients, e.g. *ABCA12, ASXL1, BCOR, CBL, CEBPA, CUX1, DNMT3A, EP300, ETV6, EZH2, FAMSC, FLT3, GNAS, HNRNPK, IDH1, IDH2, JAK2, KIT, KRAS, MLL, MLL2, MLL3, MLL5, NF1, NPM1, NRAS, NSD1, PHF6, PTPN11, RAD21, RUNX1, SF3B1, SMC1A1, SMC3, SRSF2, STAG2, TET1, TET2, TP53, U2AF1, WT1, ZRSR2*. They include epigenetic modifiers, transcription factors, spliceosome proteins, cohesins or signaling molecules. Most of them are of ambivalent significance, but some are associated with more advanced disease or progression to AML (e.g. *FLT3, IDH1, IDH2, KIT*), others with reduced overall survival (e.g. *ASXL1, ETV6, EZH2, RUNX1, TP53*) (12). Other studies (13, 14) revealed mutations in genes involved in DNA methylation or histone modifications – *TET2, ASXL1, IDH1, IDH2, EZH2, DNMT3A*. Specific effects of these mutations are unclear, but it is very likely that they are linked with epigenetic deregulation, as this kind of dysregulation is common in MDS (15).

Aim of the study

The aim of this study was to evaluate patients with suspected MDS and compare two different methods (FISH, MLPA) and their ability to detect positive samples.

Materials and methods

Samples

Samples were processed at the Department of Clinical Genetics, Medirex, a. s., Bratislava. They were collected between April 2015 and May 2016. DNA for MLPA reaction was extracted from peripheral blood or bone marrow samples collected in ethylenediaminetetraacetic acid (EDTA) by Magnesia Genomic DNA Whole Blood Kit (Anatolia Geneworks, Istambul, Turkey) according to the manufacturer's instructions and quantified with an Implen Nanophotometer (Implen GmbH, Munchen, Germany). Totally 241 DNA samples were collected from patients suspected with *de novo* MDS. Blood samples from healthy persons were used as a reference samples. The interphase FISH analysis was performed on the same group of patients, samples collected into lithium heparin tubes. There were totally 173 samples which were suitable for FISH analysis.

MLPA

The P414_MDS probemix and SALSA reagents (MRC-Holland, Amsterdam, The Netherlands) were used for MLPA reaction according to the manufacturer's instructions. The probemix contains 45 probes targeted to the specific chromosomal regions related to MDS and 1 probe designed for specific point mutation V617F of the JAK2 gene and 12 internal reference probes that are intact in MDS. Amplified probes were separated by ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, USA).

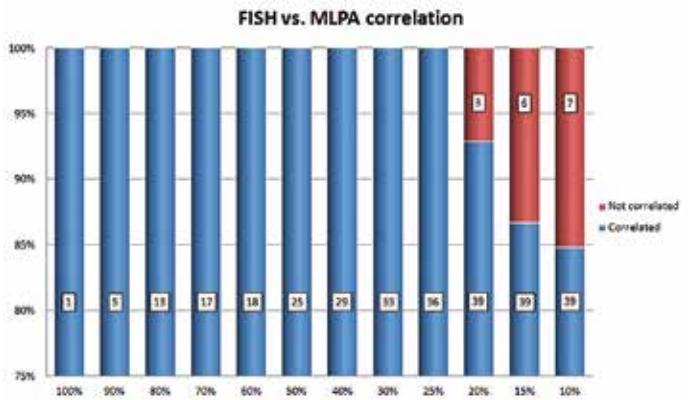
FISH

Samples collected in lithium heparin were analyzed with Metasystems probes (MetaSystems GmbH, Altlussheim, Germany) according to the manufacturer's instructions. Our MDS FISH panel included probes XL del(5q) 5p15 sg/5q31 so, XL 7q22 so/7q36 sg, XL P53, XCE Chr. 8 Blue and XL 20q12/20qter. Fluorescent signals were visualized under fluorescent microscope Olympus BX51 (Olympus, Tokyo, Japan), while at least 200 interphase nuclei were analyzed. The cut-off values were set at 5 %.

Results

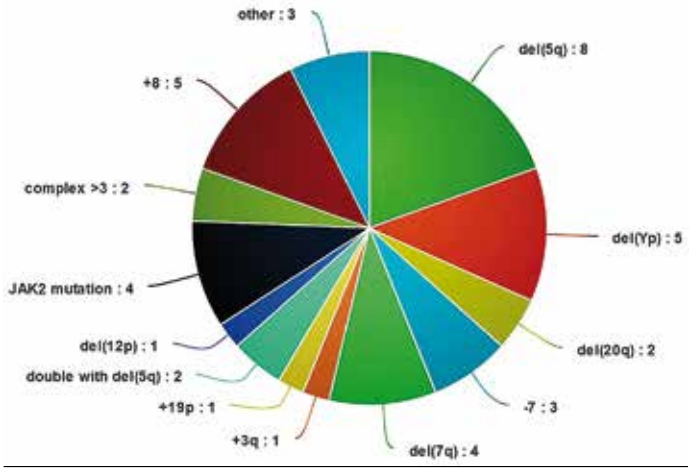
Data from ABI 3500 Genetic Analyzer were analyzed with specialized software Coffalyser.net (MRC-Holland, Amsterdam, The Netherlands). Then the results obtained from MLPA analysis were compared to the results from FISH analysis. Results from MLPA analysis showed 34 (14.11 %) positive patients, 206 negative patients (85.48 %) and only 1 uninformative

Figure 2. Correlation between FISH and MLPA results



Notes: On Y axis, there is a percentage of the correlation. There is the cut-off for FISH positivity on X axis. The numbers in columns represent the number of correlated positive aberrations (in blue columns) and non-correlated (in red columns), respectively. The number of aberrations does not correspond with the number of patients as some patients had more than 1 aberration.

Figure 3. The distribution of aberrations in our set of patients



sample (0.41 %). On the other hand, with FISH analysis 32 patients were evaluated as positive (13.28 %), 141 patients were negative (58.51 %) and 68 patients (28.22 %) were not evaluated with FISH as 4 patients (1.66 %) were uninformative, 6 failed the cultivation process and there was no suitable material available for 58 patients. The only sample uninformative in MLPA was also uninformative in FISH.

When compared, every sample negative in FISH was also negative in MLPA reaction (141/141). But there were 6 samples positive in FISH, but not in MLPA. Our observations suggest that the detection limit of MLPA reaction was around 20 – 25 % of positive cells (figure 2).

On the other hand, there were 8 patients found to be positive in MLPA, but they were not able to be evaluated by FISH due to the unavailable samples or uninformative results. In addition, there were aberrations found in MLPA which were not covered by our MDS FISH panel (+19p, del(Yp) and +3q), but they were confirmed by FISH lately. Amongst the positive samples which were not analyzed by FISH there were 4 samples positive for JAK2 V617F mutation, 1 sample with del(5q), 1 sample with del(Yp) and two samples with complex karyotype. Overall, we were able to detect several chromosome aberrations, e.g. del(5q), del(Yp), del(20q), -7, del(7q), +8, +3q, +19p, del(12p), JAK2 V617F point mutation or double aberrations with del(5q) and also 2 complex karyotypes with more than 3 chromosome aberrations (figure 3). After the exclusion of samples where FISH analysis was not performed the overall correlation between FISH and MLPA was 96.53 % (167/173).

Discussion

We compared 2 different methods which are routinely used in the diagnostics of MDS nowadays. The FISH analysis which was done on interphase nuclei and MLPA analysis which is a method based on the extracted DNA. When we compared the results, the correlation of these two methods was 96.53 %. But there were samples, which were not evaluated by FISH, because of the quality of the sample or the sample was not delivered to the laboratory in the correct medium. Amongst such samples, we were able to find 8 positive patients with MLPA analysis. In addition, MLPA was also able to find chromosome aberrations which were not covered by our MDS FISH panel of probes. On the other hand, there were also samples positive on FISH but negative in MLPA. Such findings of false negativity are in concordance with other studies (16, 17), and the reason is that MLPA analysis is not able to detect low proportion of aberrant cells. We found out that our detection limit in MLPA is about 25 % aberrant cells in the sample.

Mutation analysis of specific genes except JAK2 V617F in P414_MDS MLPA in MDS patients is not routinely diagnosed nowadays. However, by using sensitive genotyping methods in the future, such as NGS, it will soon be possible to detect many single-gene mutations simultaneously. It will help clinicians with the diagnosis, prognosis, and monitoring of MDS patients. Lately, Bejar et al. (12) proposed a molecular testing for mutations in genes *ASXL1, ETV6, EZH2, RUNX1* and *TP53*, based on their study where they demonstrated their independent prognostic value. Recent studies show the growing number of single-gene mutations involved in MDS and their prognostic significance. For example, Bejar et al. (18) showed an association between mutations in some genes and specific clinical parameters and used them to calculate the risk score for the lower-risk MDS patients.

With the continuously decreasing prices of next generation sequencing, there is no doubt that NGS testing will be used in the near future together with current methods, such as morphology, flow cytometry and metaphase cytogenetics. The advantage of NGS is that it can detect many mutations at the same time. It is not limited only to point mutations, but can also discover insertions, deletions, balanced translocations and CNVs. In addition, NGS assays can be designed only for selected panel of genes, and by multiplexing many patients can be analyzed in one reaction, which also decreases the price of diagnostics.

Conclusion

We found out that our result from FISH and MLPA correlated in 96.53 %. Both methods have their advantages and limitations, but by combining them we were able to detect more aberrations and at the same time we were able to decrease the number of false negative results. MLPA offers time-effective analytical method with relatively easy data interpretation. The powerful combination of MLPA and FISH makes a useful tool for screening for multiple aberrations in MDS patients.

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